



Original Article

A study on the genetic analysis of clinical isolates and vaccine strains of *Bordetella pertussis* by pulsed-field gel electrophoresis (PFGE)

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ABSTRACT

Considering the circulation of *Bordetella pertussis* clinical strains among populations with high vaccination coverage, it is necessary to have a proper understanding of this bacterium causing whooping cough. Various techniques, which are available for studying *B. pertussis*, can facilitate a proper comparison between different populations. We genotypically analyzed a collection of two vaccine strains used for the production of killed pertussis vaccine during 2000-2014 at Razi Vaccine and Serum Research Institute, Karaj, Iran. Ten clinical and two reference (Tohama 1 and 18323) strains were used by means of pulsed-field gel electrophoresis (PFGE). The genetic profiles of the vaccine master and working seeds showed no significant changes in the frequency of fingerprint types in the vaccine strains; also, the homogeneity of the profiles was demonstrated. However, the clinical isolates showed heterogeneity in the genetic profiles. In addition, serotyping was performed with monoclonal antisera to agglutinogens 2 and 3. Analysis of the fimbriae showed that all ten clinical strains expressed Fim3.

Keywords: *Bordetella pertussis*, DNA fingerprints, PFGE, *XbaI*

L'analyse génétique des isolats cliniques et souches vaccinales de *Bordetellapertussis* par électrophorèse en champ pulsé (ECP)

Résumé: Etant donnée la circulation des souches de *Bordetellapertussis* au sein des populations avec une couverture vaccinale élevée, une bonne compréhension de la nature de cette bactérie provoquant la coqueluche est nécessaire. Une variété de techniques ont été mises en place afin de faciliter la caractérisation des souches de *Bordetellapertussis* au sein des différentes populations. Une analyse génotypique a été menée sur une collection de deux souches vaccinales utilisées entre les années 2000 et 2014 dans la production de vaccins inactivés de la coqueluche à l'Institut de Recherche sur les Vaccins et les Sérums Razi (Karaj, Iran). Au total, 10 isolats cliniques et 2 souches de références (Tohama 1 et 18323) ont été analysés par électrophorèse en champ pulsé (ECP). Selon nos résultats, le profil génétique de la souche-mère et des souches actives ne montre aucun changement significatif dans la fréquence des types d'empreintes observée avec les souches vaccinales. Ceci reflète donc la bonne homogénéité des profils étudiés. De plus, un sérotypage a été effectué avec des antisérums monoclonaux des agglutinogènes 2 et 3. L'analyse des fimbriae a révélé que les 10 isolats cliniques exprimaient Fim3.

Mots clés : *Bordetella pertussis*, Empreintes ADN, ECP, *XbaI*

INTRODUCTION

Whooping cough, also known as pertussis, is caused by *Bordetella pertussis* and is considered a global disease. *B. pertussis* continues to circulate even in populations where high vaccination coverage of infants and children is achieved (Advani et al., 2004). This organism, which is a small Gram-negative bacterium, is recognized as the causative agent of a respiratory infection, known as whooping cough. *B. pertussis* produces many virulence factors which are responsible for the clinical features of whooping cough. The virulence factors of *B. pertussis* are generally divided into two groups, i.e., adhesions and toxins. Adhesions such as filamentous hemagglutinin, fimbriae (Fim), and pertactin (Prn) facilitate the attachment of bacteria to the host and toxins such as pertussis toxin (Ptx) and adenylate cyclase toxin (Advani et al., 2004). Factors underlying the cyclic nature of pertussis are unclear. However, the accumulation of susceptible individuals in a population to a critical threshold is considered to be a major factor in the cyclic activity of many infectious diseases (Advani et al., 2004). The observed shift in pertussis epidemiology may be a consequence of pertussis diagnosis or host-related factors such as waning vaccine-induced immunity. A third explanation involves changes in the circulating *B. pertussis* population, leading to increased virulence or resistance to vaccine-induced immunity (Advani et al., 2004). Vaccination is recognized as the best approach to overcome pertussis. Vaccination against pertussis has been available since the 1940's. Traditionally, the vaccines were whole-cell pertussis (wP) preparations as part of the combined diphtheria-tetanus-pertussis (DTP) vaccines. More recently, acellular pertussis (aP) vaccines have replaced wP vaccines in many countries (Advani et al., 2004). DTP vaccination against pertussis was first initiated in Iran in 1950 with the use of vaccines (DTwP), manufactured by Razi Institute after approval and implementation by the National Committee on Vaccination. The advantages and impacts of this type of vaccination have been reviewed and approved in previous studies, indicating the success

of detoxified and inactivated pertussis vaccine in controlling the prevalence of this condition. According to a report by the World Health Organization (WHO), DTP vaccination coverage has been estimated to be greater than 96% in Iran since 2000 (WHO, 2015). In many countries, divergence in major antigens such as Ptx, Prn, Fim2, Fim3, and tracheal colonization factor has been reported between the vaccine strains and circulating isolates (Zhang et al., 2010). Various DNA-based techniques are available for the characterization of *B. pertussis* isolates, such as pulsed-field gel electrophoresis (PFGE), IS1002-based fingerprinting (Mooi et al., 1998; Mooi et al., 2000), multi-locus sequence typing (van Loo et al., 2002), multiple-loci variable number tandem repeat analysis (VNTR) (Schouls et al., 2004; van Amersfoort et al., 2005), and recently whole-genome DNA microarray (Cummings et al., 2004; Caro et al., 2006). Application of various techniques in different analyses encumbers direct comparison of the results of different studies. At present, PFGE is the most appropriate method for DNA fingerprinting of *B. pertussis*, as it has been shown to have a greater discriminatory power in comparison with other methods, although it is difficult to perform (Mooi et al., 2000). Considering the high incidence and presence of different circulating vaccine strains in the world, it is necessary to specifically analyze the genetic patterns of these isolates through sequence analysis of virulence factors, involved in immunogenicity. Therefore, in this study, PFGE was used to compare the circulating strains in Iran using vaccine master and working seeds at Razi Vaccine and Serum Research Institute, Karaj, Iran.

MATERIALS AND METHODS

Bacterial strains and culture studies. We employed six vaccine strains and ten clinical isolates in the present study. Six vaccine strains, including 134 and 509 master and working seeds, respectively, were used to produce DTP vaccine during 2000-2014. Also, ten clinical strains were isolated from different areas of Iran (i.e., Tehran, Qom, Khuzestan, Isfahan, and East

and West Azerbaijan); the isolates ranged between 1.5 months and 10 years old. Moreover, two reference strains (Tohama1 and 18323) were used in our analysis. All *B. pertussis* isolates were confirmed by a combination of colony morphology, growth rate evaluation, Gram-staining, biochemical tests (e.g., catalase, oxidase, and nitrate reductase tests), and lack of growth on MacConkey and blood agar. The frozen bacteria were cultured onto Bordet–Gengou agar (BGA), supplemented with 20% defibrinated sheep blood and 1% glycerol, and were incubated at 35 °C for 72 h with humidity.

Serotyping. Serotyping is one of the traditional methods, used for *B. pertussis* typing. This method was developed due to the limited number of methods available for distinguishing bacterial isolates (Mooi et al., 2000). Initially, various serotypes were used to differentiate *B. pertussis* isolates. Today, a distinction is made only between serotypes 1, 2, and 3. The antigen of serotype 1 is part of *B. pertussis* lipooligosaccharide and is not suitable for discriminating between *B. pertussis* isolates, as it does not vary between these isolates. Serotyping of the isolates by the bacterial microagglutination method was performed as described by Mooi et al. (2000). In this study, serotyping was conducted by microtiter plate–based monoclonal agglutination and slide agglutination assays. For serotyping, monoclonal antibodies to agglutinogens 2 and 3, namely *B. pertussis* anti-agglutinin 2 (WHO reference reagent, anti-*B. pertussis* fimbriae 2 monoclonal antibody, NIBSC code: 04/154, UK) and *B. pertussis* anti-agglutinin 3 (WHO reference reagent, anti-*B. pertussis* fimbriae 3 monoclonal antibody, NIBSC code: 04/156) were used. The validity of the results was confirmed by using negative and positive controls. The positive control consisted of *B. pertussis* vaccine strains 509 and 134, expressing Fim2 and Fim3, respectively. The negative control consisted of deionized water; agglutination was recorded with respect to the negative control.

Preparation of DNA plugs. *B. pertussis* colonies were resuspended in 1X TE buffer (10 mM of tris-HCl

at pH=8 and 1 mM of EDTA) to obtain a bacterial suspension, containing 10^9 CFU/ml (optical density=0.66 at 650 nm). Low-melting-point Agarose was then melted in 0.5X TBE, and 150 μ l of the substance was gently mixed with 150 μ l of the bacterial suspension (1:1), which was already incubated at 45-55 °C for proper blending. Subsequently, 300 μ l of the mixture was poured into the previously identified plug mould and then cooled in a refrigerator for 20 min until being solidified. The plugs were then transferred into 1 ml of lysis buffer (0.5 M EDTA at pH=8, containing 1% sarkosyl and 2.5 μ l of proteinase K per ml of the plug) in a Falcon tube with a screw cap. The tubes were incubated overnight in a water bath at 55 °C and subsequently washed with 10 ml of 1X TE buffer at pH=8. Then, 30 ml of 1X TE and 300 μ l of phenylmethylsulfonyl fluoride (PMSF) were added, and the mixture was incubated for 1 h at 55-57 °C. The tubes were then cooled on ice, washed with 10 ml of 1X TE buffer (pH=8), and stored in 0.5 M EDTA at pH=8.

Restriction enzyme analysis of DNA plugs. The plugs were then placed in a Petri dish and cut into two parts. The slices were incubated for 3 h at 37 °C in 100 μ l of 1X buffer solution, based on the manufacturer's instructions, containing 30 U of *Xba*I. The cleaved DNA could be maintained for several weeks at 4 °C. It should be noted that fragments of less than 30 kbp could diffuse out of the plug; therefore, storage of the plugs is not recommended for very small fragments (Mooi et al., 2000).

Electrophoresis. The CHEF-DR III apparatus with a cooling module and a variable-speed pump (Bio-Rad, USA) was used in this study. Also, 1 g of the melted Agarose gel, solved in 100 ml of 0.5X TBE and 2 L of 0.5X TBE, was prepared for the CHEFIII apparatus bioproducts. The application comb was placed horizontally, and the plug slices were applied onto the teeth of the comb (at the lowest level). All the slices were fixed on the teeth of the comb with a drop of Agarose, and the comb was positioned in the gel tray with the plugs facing the direction of the migration.

The electrophoresis unit was filled with 2.2 L of 0.5X TBE, and the cooling system was set at 14 °C. Subsequently, the gel was carefully placed in the middle position in the electrophoresis apparatus. The program was set to correspond to a migration period of about 24 h. The procedural specifications were as follows: *Xba*I ramping from 5 to 6 s at 5.5 V/cm for 5 h and ramping from 8 to 35 s at 5.5 V/cm for 19 h. Lambda markers were loaded in every run and two reference strains were run on the gel.

Ethidium bromide staining. After migration, the gel was stained by being placed in a water bath, containing 1 mg/ml of ethidium bromide for 20 min, and was then rinsed gently with tap water.

Technical validation. Each run was validated by comparing the band patterns of all reference strains (18323 and Tohamal) on the gel with a given pattern of fragment size.

RESULTS

Serotyping. Based on previous findings, the fimbrial type of *B. pertussis* can change, depending on the vaccination status of the population. In non-vaccinated populations, the most prevalent serotype seems to be Fim2, whereas in vaccinated populations, the bacteria preferentially express Fim3. WHO has recommended that whole-cell *B. pertussis* vaccines should contain strains expressing Fim2 and Fim3. Monitoring of changes in Fim serotypes of fimbrial expression is recommended as a simple means for detecting changes in *B. pertussis* populations, as fimbriae could be important protective antigens. In this study, it was shown that all ten clinical strains expressed Fim3, three 509 vaccine strains expressed Fim2, and three 134 vaccine strains expressed Fim3.

Vaccine strain analysis. DNA profiles obtained from genomic fingerprinting of the vaccine and circulating strains were analyzed and compared. The PFGE profiles, obtained from vaccine strains by GelCompar II software during 2000-2014, showed two different patterns (Figure 1). As it can be observed in Figure 1, strains 1, 2, and 3 in columns

1vac, 2vac, and 3vac had Dice coefficients equal to 100%, indicating the identical genetic pattern. Also, in columns 4, 5, and 6, the Dice coefficient was 100% of the unchanged genetic pattern with major similarity to each other. Moreover, the number of the bands observed in the first *B. pertussis* group and the number of bonding patterns in the second group were 19 and 17, respectively. The Dice coefficient of up to 65% suggested that the strains in the two groups were *B. pertussis*, although they were different from each other.

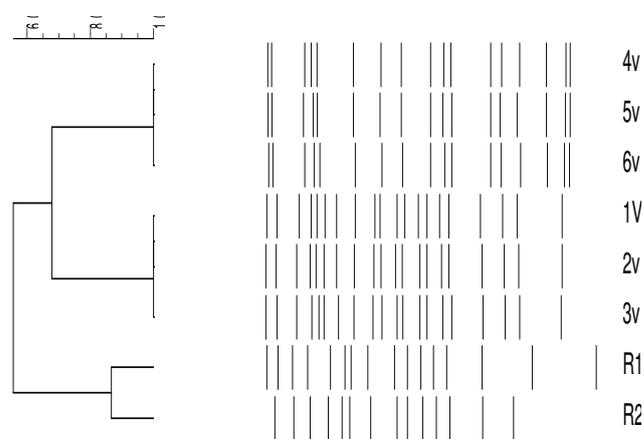


Figure 1. The dendrogram analysis of six vaccine strains (vac1-vac6) used to produce killed pertussis vaccine during 2000-2014 at Razi Vaccine and Serum Research Institute (Karaj, Iran) and two reference strains (REF1: Tohamal and REF2: 18323).

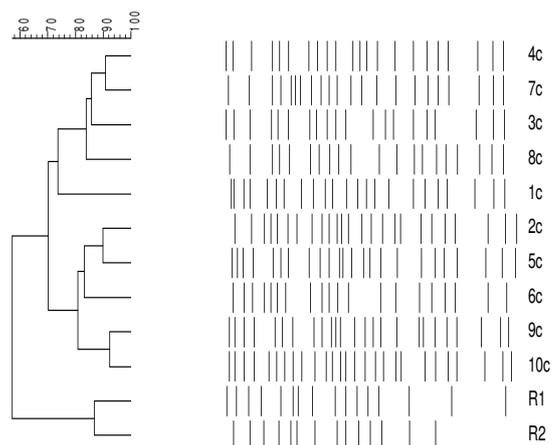


Figure 2. Dendrogram of 10 pulsed-field gel electrophoresis (PFGE) profiles of circulating *Burdetella pertussis* isolates in Iran and two reference strains (REF1: Tohamal and REF2: 18323)

Clinical isolates. The clinical strains No. 2 and 9 had Dice coefficients of approximately 94% with the isolated clinical strains No. 6 and 8, which were closely related to each other. Moreover, clinical isolates No. 1 and 5 showed approximately 87% similarity to species groups No. 2, 6, 8, and 9. The clinical isolate No. 7 exhibited 80% similarity to strains No. 1, 2, 3, 5, 6, 8, and 9. Also, strains 3 and 4 together had a similarity coefficient of approximately 83%, and their relation to both previous strains was approximately 75% (Figure 2 & 3). The clinical and vaccine strains showed heterogeneity, while the percentage of the genetic relationship was relatively high (75%).

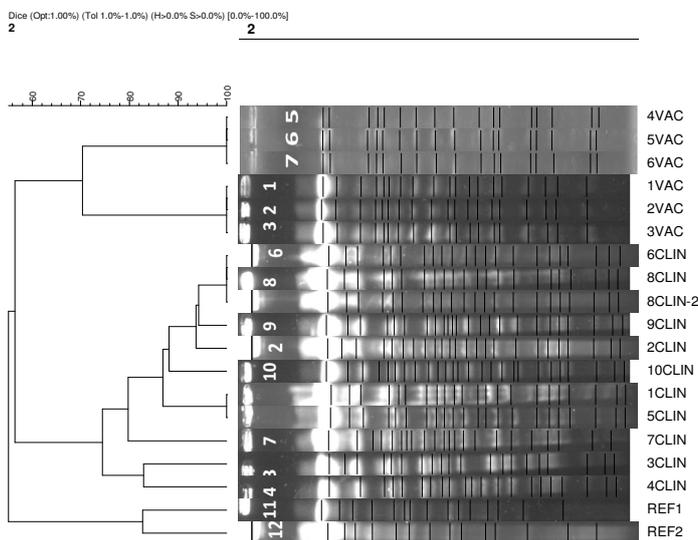


Figure 3. Dendrogram of 10 pulsed-field gel electrophoresis (PFGE) profiles of circulating *Burdetella pertussis* isolates in Iran, six vaccine strains (vac1-vac6) used to produce killed pertussis vaccine at Razi Vaccine and Serum Research Institute during 2000-2014, and two reference strains (REF1:Tohama1 and REF2:18323) using GelCompar II program

Study of comparative bonding patterns between vaccine and clinical strains. Vaccine and clinical strains showed approximately 56% similarity coefficient, which might be indicative of their association. To draw the bonding pattern graphs of the clinical isolates and vaccine strains, reference strains 18323 and Tohamal were applied by the software and

the bonding pattern was drawn. The bonding patterns of the reference strains together had an 80% similarity coefficient, and the clinical and vaccine strains together exhibited approximately 56% similarity coefficient. Vaccine and clinical strains produced distinct bonding patterns (all showing typeability patterns).

DISCUSSION

Pertussis, also known as whooping cough, is a highly transmissible disease of the respiratory system, caused by *B. pertussis* (Nar Ogun et al., 2011). Pertussis is one of the most vaccine-preventable diseases, causing morbidity and mortality in early infancy. For several decades, programs using pertussis vaccines with confirmed quality have been highly successful in preventing severe pertussis in infants, worldwide (World Health Organization, 2013). The reemergence of pertussis has raised concerns among experts. Over time, moderate changes have been observed in the gene sequence of bacterial virulence factors such as Prn and Ptx. However, epidemiological findings, which have shown that the current pertussis vaccines might gradually lose their efficacy (due to antigenic drift and continuous selection of clones with the lowest vaccine sensitivity), have not been substantiated so far (World Health Organization, 2016). In this regard, a meeting comprised of 199 representatives from different laboratories involved in epidemiological research on pertussis gathered at Pasteur Institute in Paris, France. The goal of this meeting was to reach a consensus regarding the methodology required in the epidemiological study of *B. pertussis*. Mooi et al. (2000) introduced PFGE as a standard method for studying *B. pertussis*. Overall, PFGE has become a useful tool in the study of bacterial genomes. In fact, analysis of DNA fragments, generated by rare-cutting restriction endonucleases, can be performed by PFGE. At present, the most appropriate method for DNA fingerprinting of *B. pertussis* is PFGE, as it has shown a greater discriminatory power in comparison with other methods, although it is difficult to perform (Mooi et al., 2000).

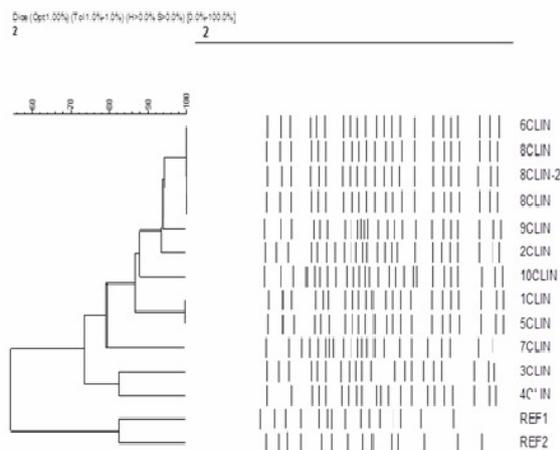


Figure 4. Clinical strain No. 8 was tested three times to demonstrate the reproducibility of the strains.

In the present study, we were able to successfully use PFGE as a tool to monitor bacterial populations in vaccine surveillance. In general, it is essential to identify key technical factors such as endonuclease selection to compare the results of isolation. Similar international studies have been conducted to evaluate the genetic map of *B. pertussis* by PFGE. In this regard, Advani et al. (2013) studied *B. pertussis* clinical isolates between 1998 and 2009 and could identify common PFGE profiles of *B. pertussis* populations, circulating in European countries with different vaccination programs and coverage (Advani et al., 2013). In addition, in a previous study in 2011, a collection of 92 clinical isolates, recovered during 2001-2009, were genotypically analyzed at the national pertussis reference laboratory through PFGE (Nar Otgun et al., 2011). Moreover, in a recent study in 13 European countries, it was shown that the type of vaccine affects the genetic changes in circulating organisms (van Gent et al., 2015). The results obtained in the present study could confirm the genetic consistency of vaccine seed strains collected during 2000-2014. Also, the procedure for assuring the quality of *B. pertussis* vaccine, using working seed cultures, showed indistinguishable changes in each strain, used

to produce killed pertussis vaccine at Razi Institute. Also, for the analysis of *B. pertussis* isolates, we only used *Xba*I restriction enzyme digestion to determine the PFGE profiles. In the current study, strains 1vac, 2vac, and 3vac had Dice coefficients equal to 100%. Also, 4vac, 5vac, and 6vac showed a Dice coefficient of 100%, which indicated an extreme level of similarity due to the absence of changes in passages for vaccine production in a period of more than one decade; also, a similar genetic pattern was observed. The number of bands in the first *B. pertussis* group was 19, while the number of bonding patterns in the second group was 17. Based on the findings, although both strain groups were *B. pertussis*, they showed a Dice coefficient of 65%, which is indicative of differences between the strains in terms of the production of specific antigens in vaccine development. Also, the clinical strains showed band differences (from 1 to 6), caused by mutations due to various factors. In general, a similarity coefficient of 100% indicates that the strains are completely identical. A similarity coefficient above 75% indicates that the isolates are part of the epidemic and are closely associated with each other. On the other hand, a similarity coefficient of less than 45% indicates that the isolates are non-related, while a similarity coefficient above 45% reveals a possible relation between the isolates. Overall, the three main characteristics of PFGE, which need to be considered, are typeability, reproducibility, and discriminatory power. In terms of reproducibility, the clinical strain No. 8 was tested three times and the results of test repetition showed the same profile (Figure 4). Based on the findings, all the strains were typeable, as it can be seen in the genetic pattern and profile of vaccine and clinical isolates. In the PFGE method, the Dice coefficient indicated the extent of the genetic relationship by using GelCompere II software for the analysis and comparison of electrophoresis patterns; therefore, this method was shown to have a high discriminatory power in the current study. We believe that this study could provide the grounds for further monitoring of vaccine strains and circulating *B. pertussis* isolates in Iran to evaluate the impact of

vaccination on genetic changes in circulating strains. It is suggested to randomly compare more isolates from different parts of Iran in order to determine the exact or whole genetic patterns of the strains. Also, it is necessary to use the most circulated genetic patterns of the strains for vaccine production in the future. In addition, we should combine other techniques in genetic fingerprinting to achieve more reliable data for analyzing vaccination programs and to prohibit vaccine import from unknown or unreliable origins with unidentified genetic fingerprinting of the strains. Through these measures, we can avoid imbalance in the circulation of vaccine strains and prevent epidemics from the current endemic status.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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